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Improvements in the gelatine-embedding technique for woodland soil and litter samples

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With 9 Figures

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1. Introduction

Many techniques have been described for the preparation of undisturbed soil sections (see Nicholas and Parkinson 1967 for a detailed review of techniques). The use of artificial resins and plastics gives excellent results for pedological studies where ultrathin sections are required (e. g. Jongerius 1963). However, the necessity for drying the samples when using these substances causes disturbances to the fine structure of the soil, particularly if a high clay fraction is present, and extensive shrinkage of organic materials occurs (Zachariae 1965). Impregnation of wet soil samples using water soluble embedding media such as agar (Haarløv and Weis-Fogh 1953) and gelatine (Minderman 1956; Vannier and Vidal 1964) has produced better results for animal and plant material. A disadvantage of these techniques is that the size of the sample is limited because of the reliance on capillarity for impregnating the sample with media.

The technique described in this paper uses vacuum impregnation. This ensures maximum penetration of air-filled interstices so that larger samples can be prepared. Some of the advantages of large samples are that disturbance at the edges is small in proportion to the size of the block, large sections are more representative of the soil and litter conditions, showing less variability in the profiles and in animal distribution from section to section. A further advantage of gelatine as an embedding medium is that arthropods can be dissected from the sections for specific identification and gut content analysis. The samples are frozen in the field with carbon dioxide ice which rapidly kills the soil and litter organisms in their natural positions and prevents disturbance to the samples between the sampling site and laboratory.

2. Methods

2.1. Sampling

Sampling methods are intended to reduce disturbance of the samples to a minimum. The corer is an open-ended cube of galvanised sheet steel with sides of 10 cm (Fig. 1, C). A piece of perforated zinc 9.8 cm square is placed on the litter surface and held lightly in position whilst the leaf litter is cut round its edges with a very sharp knife. The corer is now placed over the zinc square and is let into the soil with the knife to the required sample depth (6 cm in this case). The preliminary cutting of the leaf litter prevents lateral shearing of the litter during this process. The corer is carefully dug out and any surplus soil trimmed off. The sample is carefully pressed out into a special „embedding cage" (see below) and then packed in an insulated container with powdered CO_2 ice. After freezing the samples may be stored in a deep freeze for an indefinite period prior to embedding.

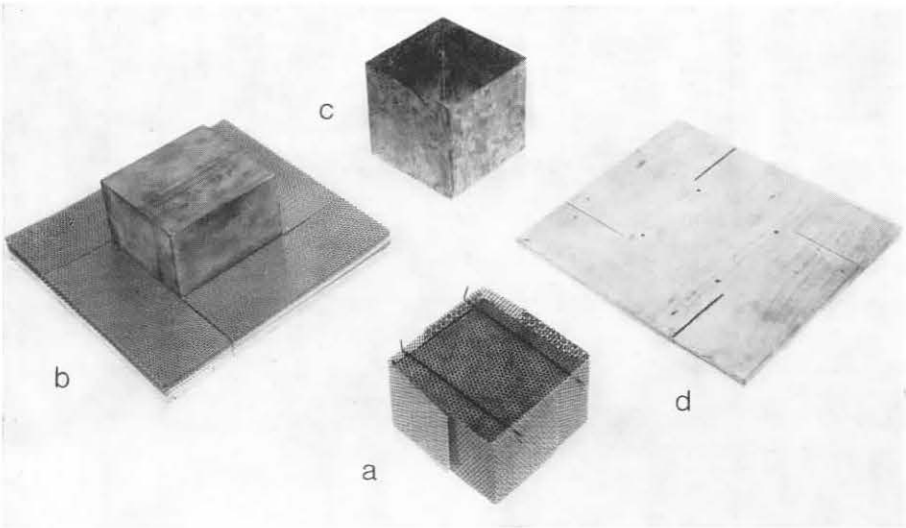

Figure 1. Equipment for sampling for gelatine embedding. (a) sample enclosed in embedding cage, (b) template/former for preparing embedding cages, (c) core sampler, (d) upper template.

2.2 The embedding process

2.2.1. *The embedding cages*

To prevent disintegration of the samples during embedding they are placed in standardised containers of perforated zinc (Figure 1a) .These are constructed using a template/former (Figure 1b) which is made in the following way. Two 23 cm square pieces of 1 cm thick ply-wood form the templates (Figure 1d) and between these are clamped a number of 23 cm square sheets of perforated zinc. Four slits are cut into the sheets using the guide slots in the templates (Fig. 2, W, X, Y, Z). A wooden block, the ‚former', 10 cm square × 6 cm (i. e. the size of the soil sample taken by the corer described above) is now clamped on top of the templates, in the position shown by dotted lines in Figure 2, and four holes (Fig. 2, A, B, C, D) are drilled through the templates and zinc sheets into the block. Nails are hammered through these holes to prevent lateral movements of the zinc sheets. Embedding cages may now be made by bending the sheets of perforated zinc up around the block. When the block is removed the sides of the cages are secured by small staples. Lids for the embedding cages are the 9.8 cm square pieces of perforated zinc placed on the litter during sampling; these are held in place by short lengths of galvanised wire passed through the perforations in the sides of the cages (Figure 1a).

2.2.2. *The embedding tank*

The embedding tank is a modified histological vacuum embedding apparatus having a controlled water bath, a 30.5 cm diameter copper tank with a heavy plate glass lid, a vacuum line attachment and air bleed. A straight sided, polythene bowl, 29 cm in diameter, is placed inside the tank as contact with copper (or other heavy metals and also high pH) inhibits the setting of gelatine. A rubber bung perforated by a short glass tube is inserted through a hole in the plate glass lid; this is connected on the inside to sufficient 4 mm bore rubber tubing to reach the bottom of the polythene bowl and to a 50 cm length on the outside. A dial vacuum gauge is coupled between the vacuum line and the tank. A water jet vacuum pump is used with the apparatus.

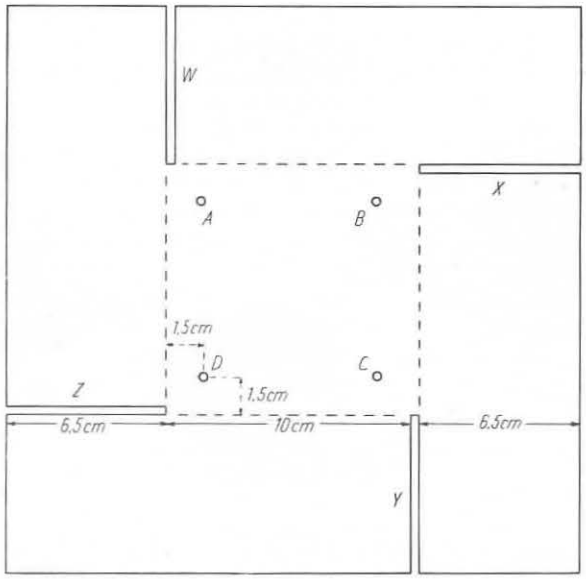

Figure 2. Design of template for the preparation of sampling embedding cages — for details see text.

2.2.3. *Embedding solutions*

A 20% solution (weight/volume) of gelatine (A.150 Technical Grade, B. Young and Co., London) is normally used at all stages of the embedding. Gelatine solutions above 10% usually cause shrinkage of soft bodied animals. However, it has been found that as the gelatine is diluted by soil water in the early stages of the embedding process, little distortion occurs.

Bacterial growths will occur in gelatine causing opalescence. Two bacteriostats have been found satisfactory, either 2% Phenoxetol (Nipa Laboratories Ltd., Pontypridd) or 1% Resiguard (Nicholas Laboratories, Slough, Bucks). Resiguard has combined fixative, softening, detergent, bacteriostatic and fungicidal properties. Phenoxetol preserves plant colours better than Resiguard but a wetting agent should be added where leaves with waxy or shiny cuticles are present.

The solutions are mixed at 55—60 °C and placed in an oven at 37 °C for a minimum of three hours to ensure complete solution of the gelatine and to allow air bubbles to rise out.

2.2.4. *Embedding, curing and sectioning*

The frozen samples are allowed to thaw and reach room temperature. The blocks are then placed in the embedding tank so that the plane of the litter layers lies vertically. This ensures first that air is not trapped between the leaves and second that if animals are displaced during embedding their movement will not affect the count for vertical distribution. The rubber tube passing through the lid is clamped and the tank evacuated to 56 cms of mercury with the water bath at 37 °C. The vacuum is maintained at a constant level by adjusting the air bleed while the inlet tube is unclamped and gelatine allowed to flow into the tank over a period of 30 minutes. When the samples are covered with 3—4 cms of gelatine the air bleed is closed and the inlet tube and vacuum line are clamped. The latter is clamped between the vacuum gauge and the water jet pump so that the pressure in the embedding tank is still registered. The samples are left under vacuum for

4—6 hours, or overnight. After this period this vacuum is released slowly, using the air bleed, to avoid disturbance as gelatine replaces any air bubbles remaining in the samples. The plate glass lid is removed and a second gelatine solution siphoned into the bottom of the bowl containing the samples, whilst removing the thin gelatine solution at the surface with a vacuum line. If the water content of the samples is 70 % or higher the gelatine is changed again after a 12 hour period using a 23 % solution. After releasing the vacuum the glass lid is replaced with a disc of expanded polystyrene to prevent condensation which causes dilution of the surface layer of gelatine. Twelve hours after the addition of the final embedding solution the polythene bowl containing the samples is lifted out of the embedding tank and cooled at 4 °C. The blocks are cut out of the gelatine, which should be firm and rubbery at this stage, removed from the embedding cages and immersed in a tank of 10 % formalin at 4 °C for 3—7 days to harden. The hardening period depends on the compactness of the samples. Finally the blocks are cut to the required thickness using a domestic rotary slicer. Sections are stored in 10 % formalin. Excellent results have been obtained sectioning down to 1 mm. Sections may be cut at 300—500 μm but a well microtome should be used, with smaller sample blocks, because thin sections of 10×5 cms are difficult to handle.

High concentrations of polyphenols are present in freshly fallen leaf litter; these are water soluble and rapidly leach out of the litter layer. HANDLEY (1954) has discussed the significance of the ability of these substances to precipitate proteins, such as gelatine, relative to the possible influence of different leaf species, with different polyphenol contents, on the formation of certain humus types. It might be expected that precipitation of gelatine would affect the process described here. In fact no gelatine precipitation occurred in the beech samples but a small quantity of flocculent precipitate was found in the litter layer of the *Castanea* samples in October when the polyphenols are at high concentration.

3. The sample freezing technique

If the distribution of soil organisms in the litter and humus profile is to be studied it is necessary to ensure that they are immobilised in the positions held during life. Rapid sampling and freezing the samples with CO_2 ice (—68 °C) kills the soil animals in situ; the effect of the low temperature is enhanced by, or follows, the narcotic effect of the CO_2 gas. The approximate rate of cooling of the samples has been determined using linked thermocouples buried in the samples and a sensitive spot galvanometer.

Samples were taken from two woodland sites with contrasting humus forms: a beech site (*Fagus sylvatica* L.) with loose, raw humus and a sweet chestnut site (*Castanea sativa* MILL) with fine, compact humus. Three samples were taken each type as for gelatine embedding, enclosed in embedding cages and placed in polythene bags. In the laboratory the samples were left to attain room temperature (about 20 °C).

Both terminals of the thermocouple were packed in CO_2 ice and the galvanometer zeroed. With one terminal in the CO_2 ice and the other in melting ice the galvanometer reading for 0 °C was recorded.

The lid was removed from one chestnut sample and one thermocouple inserted between the litter layers in the centre of the block. (The other thermocouple remained in CO_2 ice throughout the experiment.) The lid was replaced, secured and the thermocouple leads taped to the sample cage. When the galvanometer reached a steady reading the block was packed in CO_2 ice and the time recorded for the block to reach 0 °C and — 68 °C (zero deflection). These readings were repeated using the second and third samples with the thermocouple inserted between the humus and litter layers, and buried in the centre of the humus layer. The three sets of readings were repeated for the beech samples. The results are shown in Table 1.

Table 1. Cooling rate of soil samples in CO_2 ice

Position of thermocouple	*Castanea* samples (compact)		Beech samples (loose)	
	Cooling time 20 — 0 °C	Cooling time 0 — —68 °C	Cooling time 20 — 0 °C	Cooling time 0 — — 68 °C
Litter	40—50 secs	4 min	30 sec	2 min
Litter/humus	2 min	7 min	90 sec	3 min
Humus	9 min	15 min	4 min	9 min

The cooling rate of the samples is particularly dependent upon the compactness of the samples. The samples from the beech litter and humus are less compact those from the *Castanea* site and the intensely cold gas penetrates more quickly. The narcotic effect of the CO_2 appears to have considerable importance in immobilising soil organisms. Samples dissected in the field after one minute in the CO_2 ice were unfrozen in the centre,

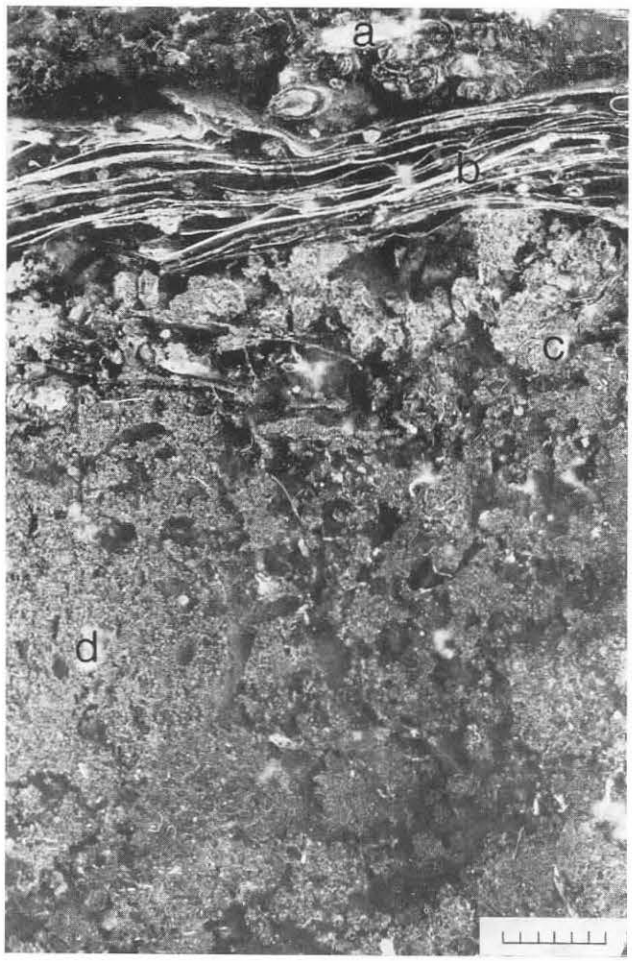

Figure 3. Section of *Castanea* site profile (4. 9. 1968). (a) *Castanea* inflorescences, (b) litter layer, (c) A_{F_2} sub-horizon, (d) A_{H_1} sub-horizon. Scale: 1 division = 1 mm.

but the soil animals were dead. Loss of active litter living arthropods may, however, occur during sampling. This may be reduced by scattering the sample area with powdered CO_2, to a depth of 5 mm. This must be allowed to sublime and the litter layers to thaw, otherwise the leaves shatter during sampling.

4. Applications of the gelatine embedding technique

The techniques described above have been developed in order to study the seasonal vertical distribution of some woodland litter and soil arthropods, in particular Oribatei (Acarina), Diplopoda and Collembola. The role played by these animals in the breakdown of litter, their feeding preferences, feeding relationships, aspects of their life cycles and microdistribution are also being studied by means of serial sections and will be the subjects of later publications.

This work is being carried out in Blean Woods National Nature Reserve, Canterbury, Kent where two dissimilar woodland soil and humus types have been selected for comparison. The first sampling area is a stand in which sweet chestnut (*Castanea sativa* MILL) dominates, which was coppiced 40—60 years ago, with a thin understorey of younger, small beech trees (*Fagus sylvatica* L.). The second area is a dense beech stand, coppiced

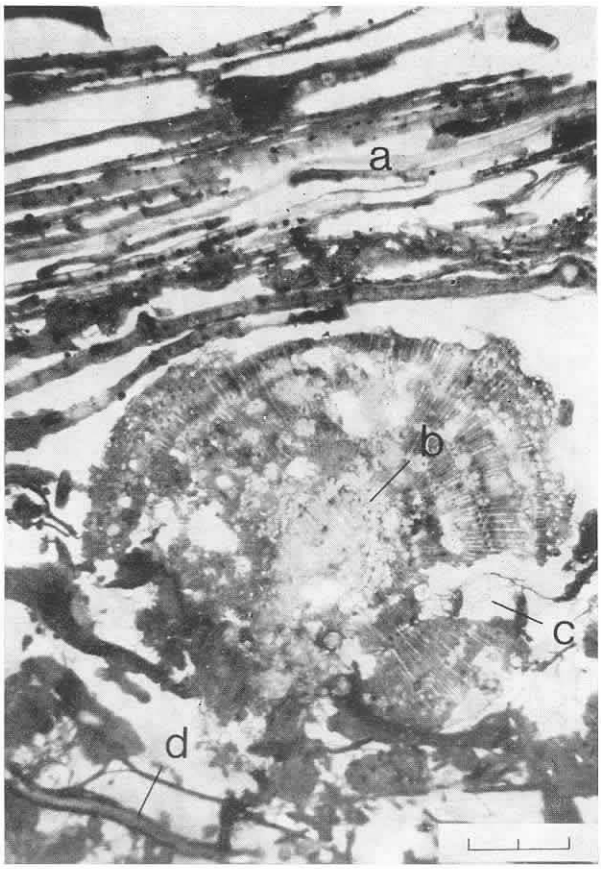

Figure 4. Section of litter layer and twig in *Castanea* site (5. 2. 1969). (a) litter layer, (b) faecal pellets of Oribatei, (c) fungal hyphae, (d) mycorrhiza. Scale: 1 division = 1 mm.

some 40 years ago, with occasional small birch (*Betula pendula* ROTH.) and oak trees (*Quercus petraea* [MATTUSCHKA] LIEBL.). Both sample sites have a complete absence of ground flora.

The litter layer of the *Castanea* site is composed, in October—November, of approximately (air dry weight) 85% chestnut, 10% beech and 5% birch and oak leaves. The humus is less than 5 cms deep and has mor-like moder characteristics. The soil is weakly podsolised with iron concentrations at a depth of 35—40 cms below a bleached alluvial sand layer and above London clay with flints.

The litter of the beech site is made up of 65% beech, 20% *Castanea* and 25% birch and oak leaves. Though lying within 20 metres of the *Castanea* sample area the humus is of a mull-like moder type, 3—7 cms in depth, on London clay with flints. The clay shows gleyisation.

The particular advantages of these sites for the study of soil organisms, apart from the absence of ground cover, is that the clay in the beech site and the sand in the *Castanea* site form barriers to the downward movement of most soil animals, except where root tracks are present. The depth of the samples taken does not extend below the 'A' horizon of the soil profile. To relate adequately the occurrence of soil organisms and signs of their activity to the litter breakdown processes it has been necessary to split the major horizon into sub-horizons based on the morphological character of the litter, and for convenience the notation of KUBIENA (1953) has been applied. The following sub-horizons are usually recognisable in samples of 6 cm depth:

Sub-horizon notation	Brief morphological description
A_O	Whole leaves showing little sign of feeding activity
A_{F1}	Fragmented leaves showing feeding by soil animals
A_{F2}	Finely fragmented leaves with raw humus
A_{H1}	Humus. Few recognisable plant remains
A_{H2}	Clay-humus complex in the beech samples
$(A_{H1}-A_E)$	Humus/bleached sand transition zone in *Castanea* samples

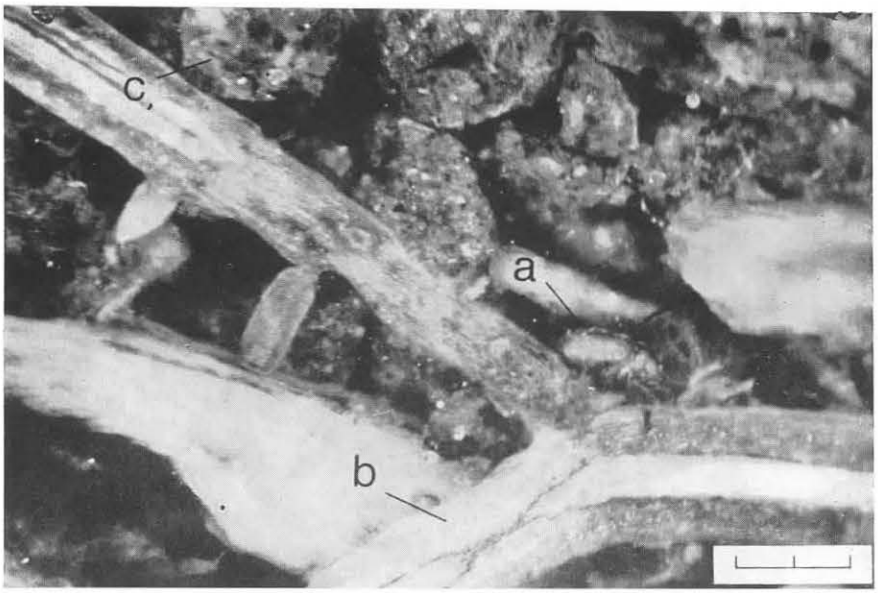

Figure 5. Detail of A_{F2} sub-horizon. (a) Oribatid mite *Nanhermannia* sp. (b) mycorrhiza, (c) earthworm faecal masses (*Lumbricus rubellus* HOFFM.), Scale: 1 division = 1 mm.

Despite the different natures of the A_{H_2} horizon in the two types of sample it is considered justifiable to include them under one heading for the purposes of this study, as they are sparsely populated with arthropods and both form physical barriers to the downward movement of most soil organisms.

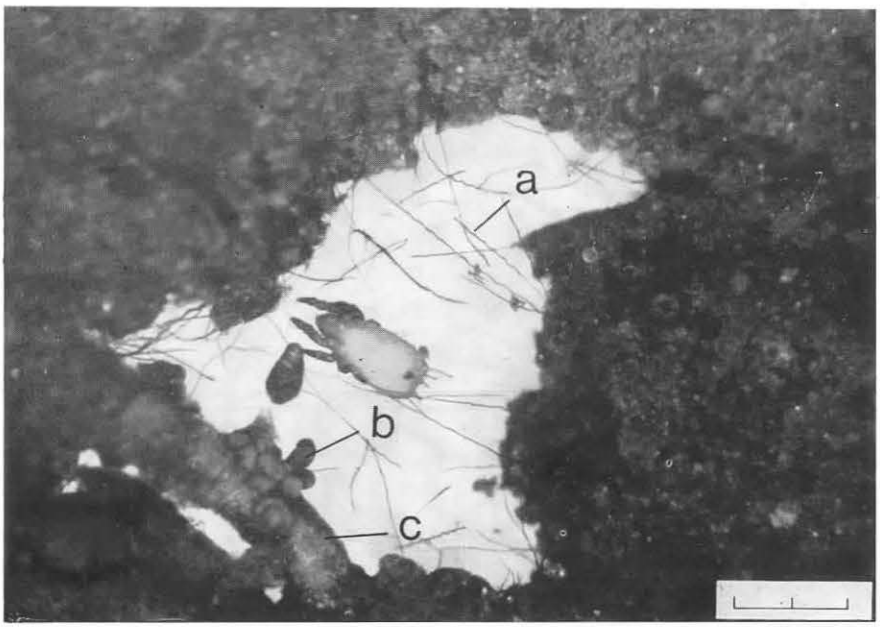

Figure 6. Oribatid nymph (*Nothrus sylvestris* Nic.) in upper A_{H_1} sub-horizon, (a) fungal hyphae, (b) Oribatid faecal pellets, (c) Mycorrhiza. Scale: 1 division = $^1/_2$ mm.

Figure 7. *Campodea staphylinus* Westwood in lower A_{H_1} sub-horizon. Scale: 1 division = $^1/_2$ mm.

4.1. Method

Three samples are taken at random each month in each of the sites. The samples are embedded, cured in formalin and sectioned into 20,5 mm slices. For viewing, sections are placed in a shallow rectangular dish, flooded with water and scanned using a stereo-binocular microscope with a gliding stage. Intense two-directional lighting is used for illumination. One face of each section is inspected. The depth of the sub-horizons is measured in alternate sections. As the depth of the litter layer is influenced by the moisture content of the leaves at the time of sampling, the number of leaves in cross-section is counted at 3 positions at 3 cm intervals across the section and the mean sectioned leaves/section recorded.

The vertical distribution of adult and juvenile Oribatei during the first six months of the study is shown in Figures 8 and 9.

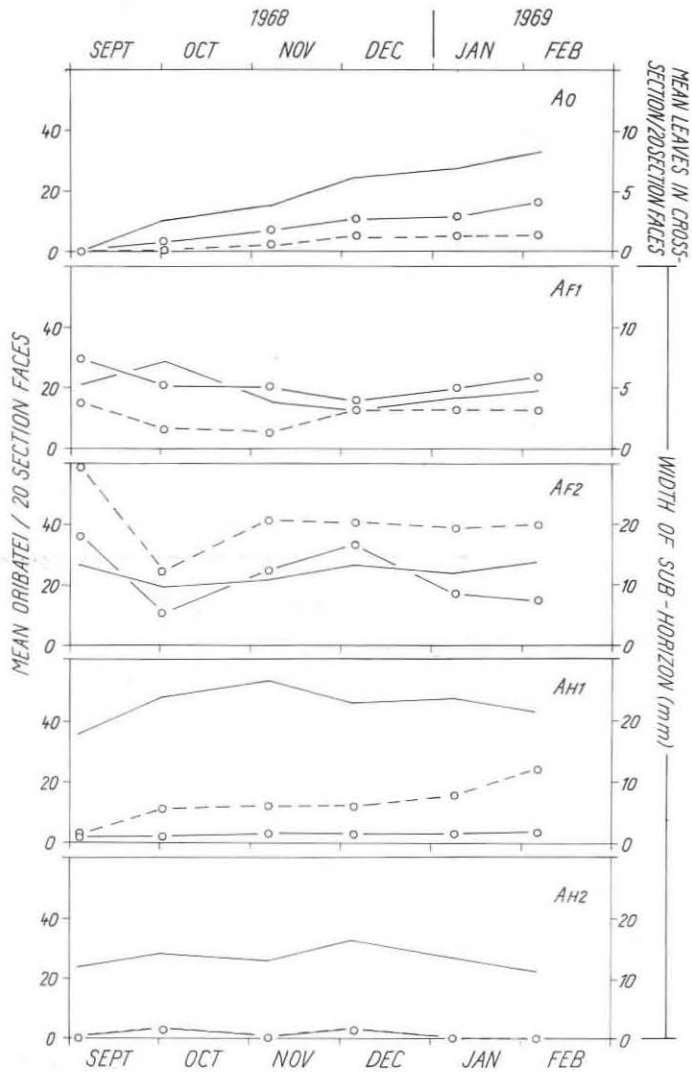

Figure 8. Vertical distribution of Oribatei in *Castanea* site.
——— width of sub-horizon
o——o adult Oribatei
o------o juvenile Oribatei

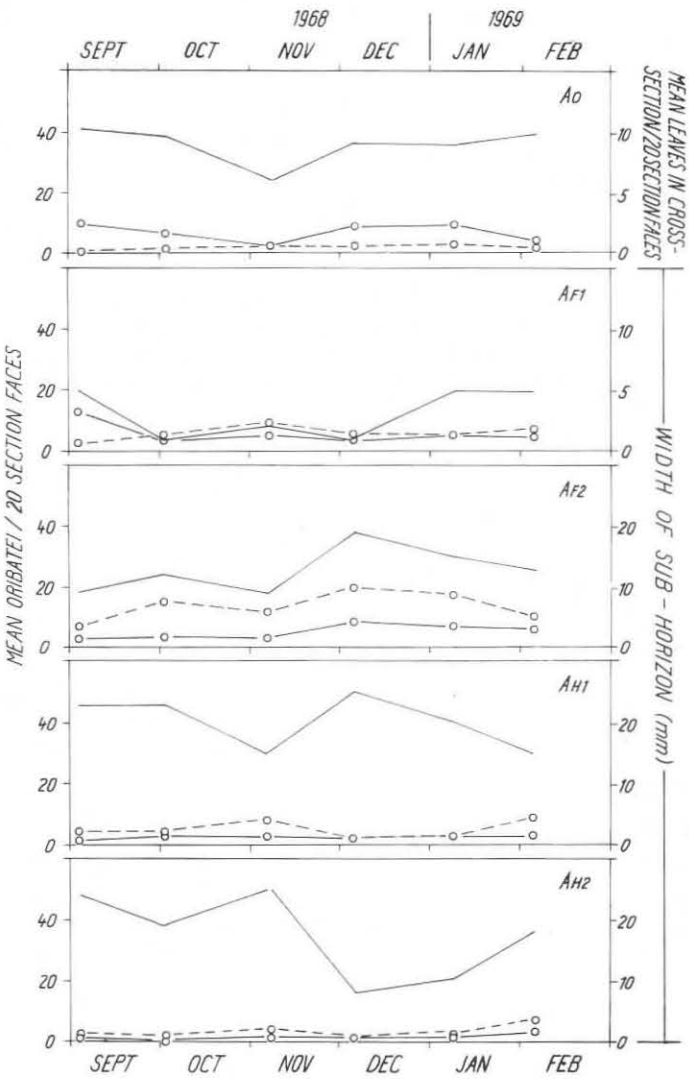

Figure 9. Vertical distribution of Oribatei in beech site
——— width of sub-horizon
o——o adult Oribatei
o------o juvenile Oribatei

5. Discussion

The main period of litter fall is the last two weeks of October; the increase in the litter layer of the *Castanea* site shown during December, January and February (Fig. 2) was due to wind shifting leaves on to the sampling area.

The rise in numbers of mites in the *Castanea* litter may be associated with the increasing complexity of the microhabitats as the litter ages, the development of the micro flora or the leaching out of substances such as tannins or polyphenols. These substances may make the freshly fallen leaves unpalatable when present in high concentrations (KING

and HEATH 1964; HEATH and KING 1967) or inhibit the digestion of the leaf material by microarthropods (FEENY 1968). It is hoped that further studies will elucidate these points. *Carabodes* species were dominant in the *Castanea* litter during the period shown and were mainly freeding on fungal hyphae.

Steganacarus magnus NIC., 1855 was the dominant adult oribatid in the beech litter (Fig. 3) during September and October. The freshly fallen litter was colonised by *Carabodes* spp. as the fungal flora developed.

The AF_1 sub-horizon is a thin layer but with relatively high populations of adult and juvenile Oribatei, particularly in the *Castanea* samples The majority of the litter feeding arthropods occur in this horizon.

The A_{F_2} sub-horizon is formed by comminuted litter and faecal material from the previous year, in the case of the *Castanea* site. In the beech site the A_{F_2} layer is formed from at least two years accumulation of finely fragmented leaf material and detritus as the beech litter is more resistant to breakdown by litter feeding animals and microflora. Mycorrhizal activity is almost completely confined to this layer in the profile. The highest population levels and greatest diversity of soil and litter organisms occur in this layer where there is wider niche diversity and microhabitat stability than in the other sub-horizons.

The activity of most Oribatei in the A_{H_1} sub-horizon is limited by the compact nature of the humus. Some Astigmatid mites, Protura, Diplura, larval and nymphal Oribatei, and Onychiuridae (Collembola) are sufficiently small to move through the pores in the humus. Immature *Schendyla nemorensis* (C. L. KOCH) (Geophilomorpha, Chilopoda) are characteristically associated with this sub-horizon. HAARLØV (1955) has quantified this relationship between soil pore size and the vertical distribution of mites and Collembola, showing that the body size of arthropods and the pore size decreases with increasing depth in the profile. In both the *Castanea* and beech samples the adult Oribatei shown for the A_{H_1} sub-horizon are mainly *Rhysotritia ardua* (KOCH) which are found burrowing in the more friable areas of the humus, or in the rhizosphere where large pore spaces are frequently associated with roots passing through the humus. Juvenile *Rhysotritia* occur in the most compact humus areas.

Most soil animals are absent from the A_{H_2} sub-horizon in both sites. This is in agreement with the observations of WALLWORK (1959) on the Oribatei and Collembola in a hemlock mor. The exclusion of animals from the mineral layer is probably due to the highly compact nature of the soil and to the quartz grains in the *Castanea* site and clay in the beech site. Added to this effect the animals in the A_{H_2} sub-horizon are likely to be limited by the available food resources. The calorific content of organic materials present in the five sub-horizons has not yet been investigated but it is unlikely that large populations of microorganisms can be supported at this level. Most of the mites occurring in this sub-horizon are larval and early nymphal stages of *Rhysotritia ardua* found tunnelling in dead roots to the full depth of the sections.

It is hoped that the use of this soil sectioning technique will enable the vertical movement of Oribatei, and other soil and litter animals, to be studied in relation to the physical conditions in the upper sub-horizons of the soil profile. This can be done with greater accuracy using sections than is possible by sub-dividing and heat extracting core samples. No significant fluctuations in the litter population of mites were found in relation to temperature and humidity during the six month period shown in Figure 8. The water content of the litter (% wet wt.) was fairly constant — $76.2 \pm 3.7\%$ in the *Castanea* site and $77.1 \pm 3.0\%$ in the beech site. Free water was present between the leaf lamellae on the six sampling dates. The microhabitats of the lower A_O sub-horizon, A_{F_1} and A_{F_2} sub-horizons at other times of the year are more stable where deep litter pockets occur as the leaves damp minor oscillations in the temperature and humidity. Temperature

records of the A_F level showed the last two weeks in December and the first week in January to be the only periods when the soil temperature fell below 0 °C (minimum temperature —2.5 °C). Normal fluctuations of different species in the oribatid population are likely to mask any vertical redistribution of mites in relation to these relatively stable winter conditions.

5.1. Comparison of the estimated Oribatei and Collembola populations by serial section and extraction methods

To obtain an estimate of the efficiency with which the sections are scanned for Oribatei and Collembola, fifteen core samples (19.6 cm² by 6 cms deep) were taken from each of the two sampling sites at the same time as the samples for embedding. The core samples had approximately the same surface area and volume as the three 100 cm² by 6 cm deep samples from each site. The core samples were divided into litter, upper 3 cm and lower 3 cm fractions and the arthropods extracted using a high gradient canister extractor similar to that described by MACFADYEN (1962). The total Collembola, *Oppia* spp., adult Oribatei and juvenile Oribatei were counted and population estimates derived. Population estimates were also derived from counts of animals in the sections, care being taken to record only those Oribatei and Collembola which were exposed on the inspected faces of the soil sections. The animals were thus effectively recorded as occurring in 20 $^1/_2$ mm sections regularly distributed through each sample block, or in approximately 1/10 of the surface area of the sample block. The three blocks have a total surface area of $\frac{100 \text{ m}^2}{3}$ so that the total number of any group of small soil animals in the three blocks (N) can be converted to a density/m² basis by the product $10N \times \frac{100}{3}$. *Oppia* spp. were not counted in the sections for this comparison as they occur mainly in the A_{F_2} and A_{H_1} sub-horizons and are extremely difficult to see, being approximately the same colour and size as many of the soil particles. The populations of Oribatei and Collembola obtained by the two methods are shown in Table 2. The population figures are not shown divided into the vertically distributed components of the samples as it is virtually impossible to divide the core samples into the same fractions as those delimited in the sections.

Table 2. Comparison of population estimates for Collembola and Oribatei derived from extracted samples and soil sections

Fauna	Beech Site Tullgren Samples Nos/m²	Soil Sections Nos/m²	*Castanea* Site Tullgren Samples Nos/m²	Soil Sections Nos/m²
Collembola	23,400	17,000	41,700	20,700
Adult Oribatei (excluding *Oppia* spp.)	22,200	19,300	39,900	50,700
Juvenile Oribatei	29,000	29,000	43,700	55,300
Oppia spp.	28,900	not counted	28,400	not counted
Total Oribatei (excluding *Oppia* spp.)	41,200	48,300	83,600	106,000

The population estimates of adult and juvenile Oribatei derived from the soil sections are 30 % higher than those from the core samples in the *Castanea* site and 20 % higher in the beech site. This may not be significant owing to the method of calculating the

population values from the soil sections, but does suggest that the use of the sections for studies of species diversity and relative abundance in different layers of the profiles (as in Fig. 8) will be justified. Further, it is of interest that higher numbers of adult and juvenile Phthiracaridae were recorded in the sections than were represented in the extracted samples. This is probably related to the endophagous habit of many juvenile Phthiracaridae, some adult *Rhysotritia ardua* and occasionally *Steganacarus spinosus*. *Rhysotritia*, as mentioned above, burrows in the humus and these animals, together with those in rotten twigs and leaf petioles may not be able to escape before desiccation during heat extraction. In this connection it was noted that approximately 12% of the juvenile Oribatei occurred in confined spaces from which their extraction was unlikely. *Hermaniella granulata* (Nic) (*Hermaniellidae*) was also occasionally found completely enclosed in rotten wood in the A_{F_1} sub-horizon. A large proportion of the juveniles of this species are also found in decayed wood and bark so that some individuals probably reach maturity under these confined conditions and then emerge to take up a freeliving existence. The soil sections give low population estimates for Collembola. This is thought to be due to the difficulty of distinguishing the many small juvenile forms present in the humus layer (A_{H_1}); these distort in the embedding process more than juvenile Oribatei of comparable size. It would be of value to embed and section extracted samples to establish the efficiency of some heat gradient extractors, but this has not yet been attempted.

6. Acknowledgements

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